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$p19^{ARF}$ Determines the Balance between Normal Cell Proliferation Rate and Apoptosis during Mammary Gland Development

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This study demonstrated, for the first time, the following events related to $p19^{ARF}$ involvement in mammary gland development: 1) Progesterone appears to regulate $p19^{ARF}$ in normal mammary gland during pregnancy. 2) $p19^{ARF}$ expression levels increased sixfold during pregnancy, and the protein level plateaus during lactation. 3) During involution, $p19^{ARF}$ protein level remained at high levels at 2 and 8 days of involution and then, declined sharply at day 15. Absence of $p19^{ARF}$ in mammary epithelial cells leads to two major changes, 1) a delay in the early phase of involution concomitant with downregulation of $p21^{Cip1}$ and decrease in apoptosis, and 2) $p19^{ARF}$ null cells are immortal in vivo measured by serial transplantion, which is partly attributed to complete absence of $p21^{Cip1}$ compared with WT cells. Although, $p19^{ARF}$ is dispensable in mammary alveologenesis, as evidenced by normal differentiation in the mammary gland of pregnant $p19^{ARF}$ null mice, the upregulation of $p19^{ARF}$ by progesterone in the WT cells and the weakness of $p21^{Cip1}$ in mammary epithelial cells lacking $p19^{ARF}$ strongly suggest that the functional role(s) of $p19^{ARF}$ in mammary gland development is critical to sustain normal cell proliferation rate during pregnancy and normal apoptosis in involution possibly through the p53-dependent pathway.

INTRODUCTION

One important control mechanism of cell growth depends on the tumor suppressor *p53* gene, whose inactivation is the most frequent event in human cancer (Levine, 1997). A second control mechanism, which prevents cells from indefinite proliferation, is governed by the CDK inhibitor Cdkn2a (also known as $p16^{INK4a}$; Serrano *et al.*, 1993; Ruas and Peters, 1998), whose concentrations rise with accumulating population doublings. Binding of Cdkn2a ($p16^{INK4a}$) to cdk4 and cdk6 (Serrano *et al.*, 1993) inactivates cdk kinase activities and induces a G1-phase cell cycle arrest by preventing the inactivation of the tumor suppressor RB1. Cdkn2a ($p16^{INK4a}$) is encoded by the *CDKN2A* (*INK4A/ARF*) tumor suppressor locus (Serrano *et al.*, 1993; Sharpless and DePinho, 1999).

Cdkn2a also controls the p53 pathway by generating an alternative transcript that encodes Cdkn2a ($p14^{ARF}$) in humans or Cdkn2a ($p19^{ARF}$) in mice (Quelle *et al.*, 1995; Kamijo *at al.*, 1997; Dimri *et al.*, 2000). $p19^{ARF}$ sequesters the oncoprotein Mdm2 to the nucleolus (Pomerantz *et al.*, 1998; Zhang and Xiong, 1999; Weber *et al.*, 2000b) and blocks nucleocytoplasmic shuttling of MDM2 (Tao and Levine, 1999; Zhang and Xiong, 1999). This prevents p53 degradation by MDM2 and leads to increased p53 stability and function in the nucleoplasm (Zhang *et al.*, 1998; Tao and Levine, 1999; Zhang and Xiong, 1999).

Recently, $p19^{ARF}$ has been reported to interact with targets other than Mdm2 to inhibit cell proliferation by a mechanism that is independent of p53 (Weber *et al.*, 2000a). These proteins are E2F-1, E2F-2, and E2F-3, and their binding to $p19^{ARF}$ results in destabilization and degradation of these molecules and suppression of cell proliferation (Eymin *et al.*, 2001; Martelli *et al.*, 2001). Furthermore, $p19^{ARF}$ was found colocalized with DNA replication protein A, a protein critical in DNA synthesis, within the nuclear bodies, impeding DNA synthesis (Yarbrough *et al.*, 2002). Additionally, in primary cells, E1A, RAS, ARAF1, Myc, Abl, and E2F1 elicit an apoptotic response or a senescence-like growth arrest by inducing $p19^{ARF}$ and/or $p16^{INK4a}$ expression (Serrano *et al.*, 1997; de Stanchina *et al.*, 1998; Zindy *et al.*, 1998; Cong *et al.*, 1999; Dimri *et al.*, 2000; Russell *et al.*, 2002).

The *INK4A/ARF* locus is frequently found deleted or silenced in many types of tumors and cell lines, thus, inactivating both the $p16^{INK4a}$/pRB and the $p19^{ARF}$/p53 pathways through a single event, which suggests that inactivation of these genes is a critical step for tumorigenesis. Genetic and epigenetic analysis of $p14^{ARF}$ mutation, homozygous and hemizygous deletion, and meththylation cumulatively affect ~41% of the 100 primary breast carcinomas. Furthermore, TBX2, a potent immortalizing gene that represses the 14^{ARF} promoter was found amplified in a subset of primary human breast tumors (Jacobs *et al.*, 2000). Interestingly, there were no detectable genetic alterations observed in the majority of cases with overexpressed $p14^{ARF}$ mRNA (Silva *et al.*, 2001). Emerging evidence suggests that the broad inhibitory role of $p19^{ARF}$ through the p53-dependent and -independent pathways may serve to counteract many oncogenic signals in breast epithelium.

In respect to mammary gland development, it is unknown how $p19^{ARF}$ is regulated and functionally involved in normal mammary gland development, what are the down-

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stream events upon loss of p19^{ARF} function, and whether loss of p19^{ARF} imparts an increased risk for immortalization and tumorigenesis to mammary epithelial cells to in vivo. In this study, we utilized the p19^{ARF} knockout mouse model to answer these questions. Results from this study have provided, for the first time, data demonstrating that p19^{ARF} is upregulated by progesterone and that p19^{ARF} is a critical molecule to maintain normal rate of cell proliferation and apoptosis during pregnancy and involution, respectively. Loss of p19^{ARF} leads to profound downregulation of p21^{Cip1} and immortalization of mammary epithelial cells in vivo.

MATERIALS AND METHODS

Mice

A pair of male and female p19^{ARF} heterozygous mice (129s*vj* × C57BL6) background was obtained from Drs. M. Roussel and C. Sherr in the Departments of Genetics and Tumor Cell Biology at St. Jude Children's Research Hospital and HHMI, respectively, in Memphis. Mice progeny were genotyped by PCR as described earlier (Kamijo *et al.*, 1997). All experiments in this study were performed utilizing 129s*vj* × C57BL6 background mice unless indicated.

Morphogenesis and Histology Analysis

Two hours before sacrifice, all mice were injected with 70 μg/g body weight bromodeoxyuridine (BrdU) cell proliferation labeling reagent (Sigma, St. Louis, MO). Number 4 inguinal mammary glands were removed from four to six mice. The left inguinal mammary glands were processed for whole mounts for morphogenesis analysis and the right inguinal mammary glands were fixed in 4% paraformaldehyde at 4°C overnight followed by 70% ethanol. Tissues embedded in paraffin were sectioned and stained with hematoxylin and eosin for histological analysis.

Immunostaining for cell proliferation utilized the BrdU cell proliferation kit from Amersham Biosciences Inc. (Arlington Heights, IL) and apoptotic activity utilized In Situ Apoptosis Detection kit from Trevigen (Gaithersburg, MD). For each tissue section, a random field of 1000 cells was counted. The average number of positive BrdU staining cells or apoptotic cells in a given tissue section was obtained by taking the average obtained from counting three separate glands of 1000 cells per section.

RT-PCR and Western Blot Analysis

Mammary glands from WT and p19^{ARF} null mice at 12 weeks' virgin, midpregnant, 10 days' lactation, and 2, 8, and 15 days of involution were homogenized in lysis buffer containing Tween 20, and protein was quantified by BCA assay (Pierce, Rockford, IL). Equal amounts of protein extract (500 μg/sample) were resolved by 12.5% SDS-PAGE and transferred, and membranes were probed with p19^{ARF} polyclonal antibodies (Novus Biologicals Inc., Littleton, CO). The membrane was developed by ECL chemiluminescent detection reagents (Amersham Pharmacia Biotechnology, Piscataway, NJ). The membrane was stripped and reprobed with mouse mAb against actin (Santa Cruz Biotechnology, Santa Cruz, CA) for equal sample loading. Protein bands were scanned and quantified by PhosphoImager SP scanner (Molecular Dynamics, Sunnyvale, CA). The p19^{ARF} protein bands were normalized to the actin protein level.

We chose to utilize the RT-PCR method to measure p19^{ARF} message levels instead of Northern blot analysis during mammary gland development, because the signal was rather low and not accurately reproducible. The RT-PCR for p19^{ARF} was performed on polyadenylated mRNA isolated from total RNA for higher sensitivity and accuracy. Total RNA was isolated from WT mammary glands at the same stage-points of development indicated for the protein level using RNA-Trizol reagent as described by the manufacturer (Invitrogen, Carlsbad, CA). The poly (A) mRNA was isolated from total RNA by Oligotex mRNA Kits (QIAGEN, Chatsworth, CA). The test samples and the standard dilutions were prepared in duplicates at the same time and conditions using RiboGreen RNA Quantitation Kit (Molecular Probes, Eugene, OR) for a better accuracy and sensitivity measurement of mRNA concentration. The standards and test sample concentrations were measured using Microplate Flurescence Reader, FLx800 (Bio-Tek Instruments, Burlington, VT). Equal amounts of poly (A) mRNA (300 ng) per sample were reverse-transcribed according to the manufacturer using SuperScript one step RT-PCR system (Invitrogen Life Technologies). At the end of RT reaction at 50°C for 30 min, 1 μl was removed for the β-actin PCR using primers specific for actin (Sigma-Genosys, The Woodlands, TX). The 19 μl was used for PCR amplification of p19^{ARF} using specific primers for p19^{ARF} transcript, as previously described (Kamijo *et al.*, 1997). Both β-actin and p19^{ARF} were amplified using 35 cycles of denaturation (95°C, 1 min), annealing (65°C, 50 s), and extension (72°C, 1 min). Products (10 μl per lane) were electrophoresed on 1.8% agarose gels, visualized by ethidium bromide staining, analyzed with an Alpha Innotech imager (San Leandro, CA), and quantified with ImageQuant software (Madison, WI).

Double-immunoflorescent Staining and Confocal Imaging

To examine p19^{ARF} cellular localization, mammary glands were removed from the animals, embedded in Cryo-gel, and sectioned at 50 μm using a microtome cryostat. The thick frozen sections were mounted onto positively charged slides, permeabilized in 0.5% Triton X-100 in PBS for 5 min at room temperature, and fixed in 4% formaldehyde in PBS. Tissue sections were blocked in 5% bovine serum albumin in PBS followed by incubation with (1:100 dilution) of p19^{ARF} rabbit polyclonal antibody (Novus Biologicals Inc.) overnight at 4°C and then with (1:1000 dilution) of goat anti-rabbit/Texas Red (Molecular Probes). Tissue sections were washed in PBS and then incubated with (1:500 dilution) mouse keratin 14 (K14) mAb followed by incubation with (1:500 dilution) of goat anti-mouse/FITC (Molecular Probes) and were washed and mounted. The keratin staining is specific for myoepithelial cells. Immunofluorescence microscopy was performed using confocal microscope (Molecular Dynamics; SARASTRO 2010). Several layers of each mammary gland section were studied and photographed to show localization of p19^{ARF} and K14 at different conditions of hormonal stimulation.

Stimulation of Mice with Individual or Combination of Hormones

To determine the appropriate time point to collect mammary glands for p19^{ARF} protein level detection after stimulation of mice with individual or a combination of hormones, a preliminary experiment was performed on a cohort of WT virgin mice at 12 weeks of age, each received a pituitary isograft at the same time. Mammary glands were removed at 0 (untreated), 5, 11, and 15 days after the pituitary gland had been implanted. Mammary glands were analyzed for morphogenesis in whole mounts as described earlier (Lydon *et al.*, 1999) and for p19^{ARF} protein level by Western blot analysis at same indicated time points. Based on data of this experiment, mammary glands were collected 15 days after individual or a combination of hormone stimulation for p19^{ARF} protein analysis.

Individual or combination steroid hormones (50 μg estradiol benzoate and 20 mg progesterone) in silastic tubing were administered and resulted in a constant systemic release of hormones (Guzman *et al.*, 1999). WT virgin mice at 12 weeks of age were implanted with silastic tubing subcutaneously in the upper back. Prolactin (PRL; NIH source) was administered by subcutaneous injection, 1 μg/g body weight, twice daily, based on protocol in previous studies (Horseman 1999; Vonderharr, 1999). The fifth group was untreated (control) age-matched mice. Mammary glands of all five groups are collected 15 days after the tubing implants. Mammary glands were analyzed for morphogenesis, p19^{ARF} localization, and protein level.

Analysis of p19^{ARF} Null Mammary Gland Transplants

Serial transplantation experiment was performed on BALB/c p19^{ARF}-null mice to examine the immortalization of p19^{ARF} null mammary epithelial cells in vivo. The p19^{ARF} null genotype was backcrossed into BALB/c mice for nine backcross generations. The BALB/c mice were chosen for this experiment for the following reasons: 1) the susceptibility of mammary epithelium to neoplastic transformation is much greater upon the BALB/c than the C57Bl/6 background, 2) the studies examining other genes in this assay have been done on the BALB/c background (i.e., p53); therefore, it is more amenable to make comparisons on the role of a specific gene in the events of both immortalization and neoplastic transformation, and 3) the transplantation of the epithelial cells is more readily accomplished in a recipient strain with a single genetic background than a chimeric background.

The procedures for clearing and transplanting the mammary fat pads are standard and were followed as described earlier (Young 2000). Briefly, mammary glands dissected randomly into fragments and mammary ducts (~1–2 mm^2) in replicates (n = 6) used for both WT and p19^{ARF} null transplantation. Using dissecting microscope, a pocket in the middle of the left and right side of the cleared fat pad no. 4 were prepared. A 1–2-mm^2 WT ductal mammary tissue was transplanted into the left no. 4 mammary fat pad and p19^{ARF} null mammary epithelium into the right no. 4 mammary fat pad of WT recipient mice (n = 6). At 8 weeks' posttransplant, the outgrowths were examined and one fat pad of each, WT and p19^{ARF} null, were used as donors for the next generation. The donor fat pads and the other remaining fat pads in that generation were then whole-mounted and examined for morphology and extent of growth (percentage of fat pad filled). On the 6th transplant generation, outgrowths of p19^{ARF} null and WT, as a control, were examined for cell proliferation by BrdU labeling, morphogenesis, and percent of fat pad filled by whole mounts, as well as p53, p21^{Cip1}, p27^{kip1}, and p16^{INK4a} by immunohistochemical staining. Antibodies used are rabbit polyclonal antibodies against p53, p27^{kip1}, and p16^{INK4a} (Santa Cruz Biotechnology) and anti-mouse p21^{Cip1} (PharMingin Laboratories, San Diego, CA).

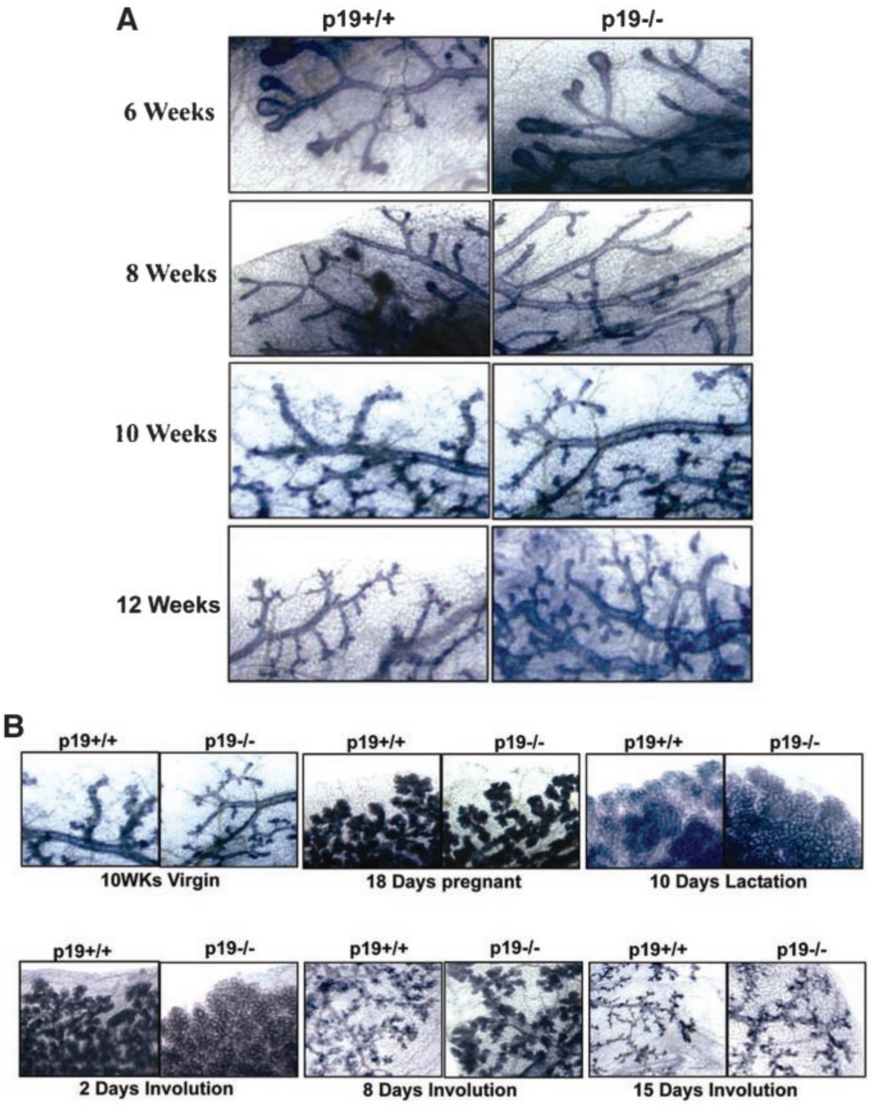


Figure 1. Morphogenesis of WT and p19^{ARF} null mammary glands during development. Parts of whole mounts of WT and p19^{ARF} null mammary glands showing the morphological differences and similarity in both groups during development. (A) Various time points of virgin mammary gland during development and (B) stages of mammary gland development at indicated stage-points.

RESULTS

Morphogenesis of Mammary Gland in the Absence of p19^{ARF} during Development

We examined at least four mammary glands of WT and p19^{ARF} null mice for morphogenesis at the following stages during development: 6, 8, 10, and 12 weeks of age (virgin), midpregnant, 10 days' lactation, and 2, 8, and 15 days of involution. The p19^{ARF} null mammary glands in virgin mice revealed slightly dilated primary branches and moderate increase in tertiary branches at 10 and 12 weeks of age compared with WT (Figure 1A). There were no differences in morphogenesis between WT and p19^{ARF} null during pregnancy and lactation. However, differences in development were most apparent at 2 and 8 days of involution with a marked delay in involution at in the p19^{ARF} null mammary glands (Figure 1B). These data indicate two points: 1) p19^{ARF} null mammary epithelial cells undergo normal alveolargenesis during pregnancy; and 2) a delay in involution at 2 and 8 days, suggesting that p19^{ARF} may play a critical role in inducing apoptosis in the first days (1–8 days) of involution, perhaps through p53-dependent pathways.

Cell Proliferation and Apoptosis in the Absence of p19^{ARF} during Mammary Gland Development

To confirm if p19^{ARF} regulates cell proliferation or/and apoptosis during mammary gland development, staining for BrdU and apoptotic cells was performed. Cells containing BrdU immunoreactivity were detected at equivalent levels in adult virgin WT and p19^{ARF} null mice at 12 weeks of age (Figure 2A). The rate of cell proliferation increased significantly ($p < 0.05$) in mammary glands of p19^{ARF} null mice compared with the WT in the stage of pregnancy. At 2 days of involution, the rate of cell proliferation increased four- and twofold in WT and p19^{ARF} null mice, respectively, compared with their counterparts at 10 days' lactation (Figure 2A). In WT, cell proliferation declined sharply at 8 and 15 days of involution, but continued to increase significantly ($p < 0.023$) at 8 days of involution in the p19^{ARF} null mammary glands compared with WT (Figure 2A). In p19^{ARF} null mammary gland, cell proliferation declined twofold compared with the 8-day time point, but was still twofold higher than WT at 15 days. These data clearly suggest that mammary epithelial cells are responding differently to proliferative stimuli in the presence or absence of p19^{ARF} during development.

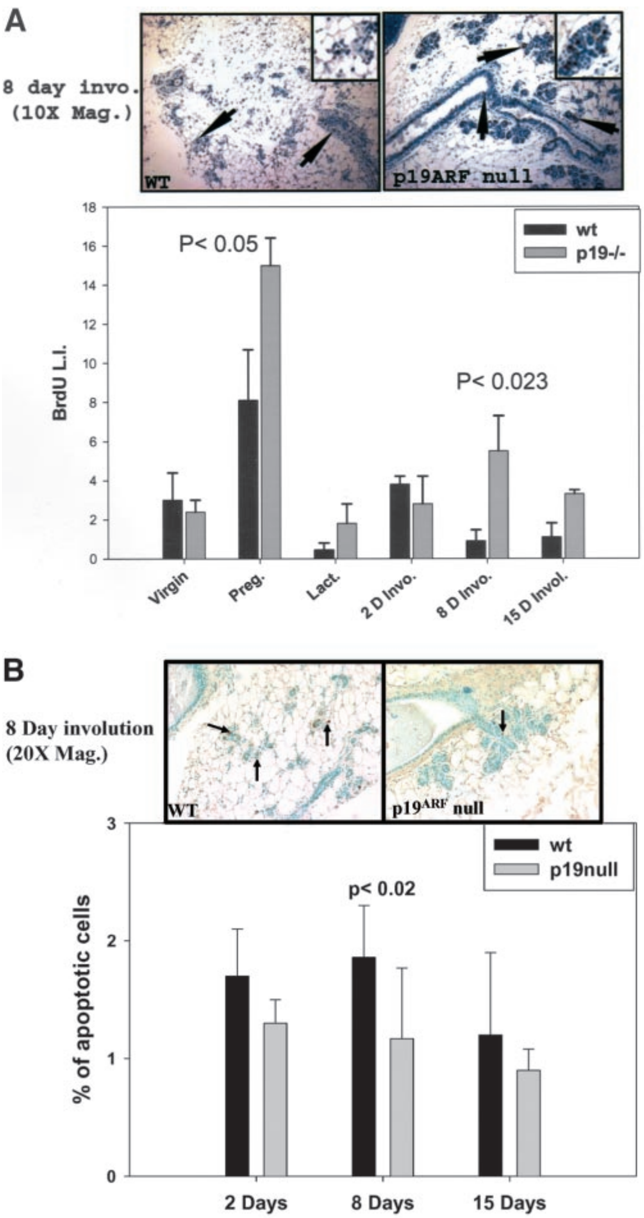


Figure 2. Rate of cell proliferation and apoptosis in WT and p19^{ARF} null mammary glands during development. (A) Rate of cell proliferation presented as mean percent of BrdU LI ($\pm$SD) in a bar graph calculated from 4–6 mammary gland/stage-point in each group and statistically analyzed using the two-sided Student's *t* test. On the top of the bar graph, examples of mammary gland sections showing the proportion of number of cells stained positively for BrdU in WT vs. p19^{ARF} null at 8 days of involution. (B) Bar graph showing mean percentage apoptotic cells ($\pm$SD) in WT and p19^{ARF} null mammary glands at indicated time points during involution. On the top, examples of mammary gland sections showing the proportion of number of positive apoptotic cells stained in WT vs. p19^{ARF} at 8 days of involution ($p < 0.02$).

The function of p19^{ARF} is well documented through the p53 pathway in cell growth arrest and apoptosis in many cell types by sequestration of Mdm2 to prevent p53 degradation (Kamijo *et al.*, 1998; Pomerantz *et a*l., 1998; Zhang *et al.*, 1998; reviewed in Sherr 1998; Tao and Levine, 1999; Weber *et al.*, 1999; Zhang and Xiong, 1999). However, it is unclear whether absence of p19^{ARF} in mammary epithelium would delay cell regression through reducing apoptosis during involution. In a recent report, an increase in p19^{ARF} was demonstrated in WT BALB/c mice at 2 days of involution concomitant with p53 activation (reviewed by Jerry *et al.*, 2002). p19^{ARF} was also implicated in inducing p53 acetylation that led to its activation (Korgaonkar *et al.*, 2002). The difference in the total number of apoptotic cells was apparently high in WT at all time points during involution, but not significant, compared with p19^{ARF} null mammary glands, except at 8 days, where the percent of apoptotic cells was lower ($p < 0.02$) in the p19^{ARF} null cells (Figure 2B). These data suggest that p19^{ARF} may be involved in p53-dependent apoptotic activities, and its absence may interrupt p53 activation, resulting in a delay in involution. The significant differences in cell proliferation and apoptosis between WT and p19^{ARF} null mice may indicate a critical role for p19^{ARF} at around day 8 of involution.

The Pattern of p19^{ARF} Expression during Normal Mammary Gland Development

The normal mammary gland undergoes a series of morphological changes that are attributed to changes in steroid hormones and growth factors during development. To understand the interplay between intracellular and extracellular factors that regulates mammary epithelial cell proliferation, differentiation, and apoptosis in relation to p19^{ARF} during development, 19^{ARF} protein and RNA levels were determined in WT mice. It is well known that p19^{ARF} is a weak or undetectable in quiescent cells and its expression increases in proliferating cells upon mitogenic signals (Ruas and Peters, 1998; reviewed by Sherr, 1998). After experimental optimization, 500 μg of protein extract/sample was utilized to measure the levels of p19^{ARF} by Western blot analysis during development. The p19^{ARF} protein level was undetectable in 12-week virgin mammary gland, increased sixfold at midpregnancy, when cell proliferation and differentiation are maximum, and remained at the same level during lactation (Figure 3A). During involution, a slight increase was observed in p19^{ARF} protein levels at 2 and 8 days, followed by a sharp decline at 15 days (Figure 3A). The mean value of p19^{ARF} protein level at 2 and 8 days of involution was statistically higher ($p < 0.0012$) than its mean value of pregnant and lactation together. Based on these data, it is possible that p19^{ARF} functions are required during pregnancy, lactation, and the first 8 days of involution.

To assess whether p19^{ARF} mRNA expression responds to alterations during mammary gland development, similar to the protein levels, RT-PCR analysis of p19^{ARF} message levels was performed on the same stages of development. Although RT-PCR is not a quantitative analysis, nonetheless utilizing two animals per stage-point and normalization of data with β-actin message level measured in the same RT reaction mixture has allowed us to be able to compare p19^{ARF} message levels during development. The p19^{ARF} message level was low in mammary gland of 12-week-old virgin mice, increased eightfold in midpregnant mice, and declined at 10-days lactation to same level as in virgin mice (Figure 3B). During involution, the p19^{ARF} message level was induced profoundly at all time points during involution (Figure 3B). The mean value of the message level of p19^{ARF} increased insignificantly during involution compared with the stage of pregnancy. These data suggest the following: 1) both p19^{ARF} protein and expression levels were induced during pregnancy and involution, which indicates that p19^{ARF} is regulated by hormone and nonhormone regulators during mammary gland development; and 2) the con-

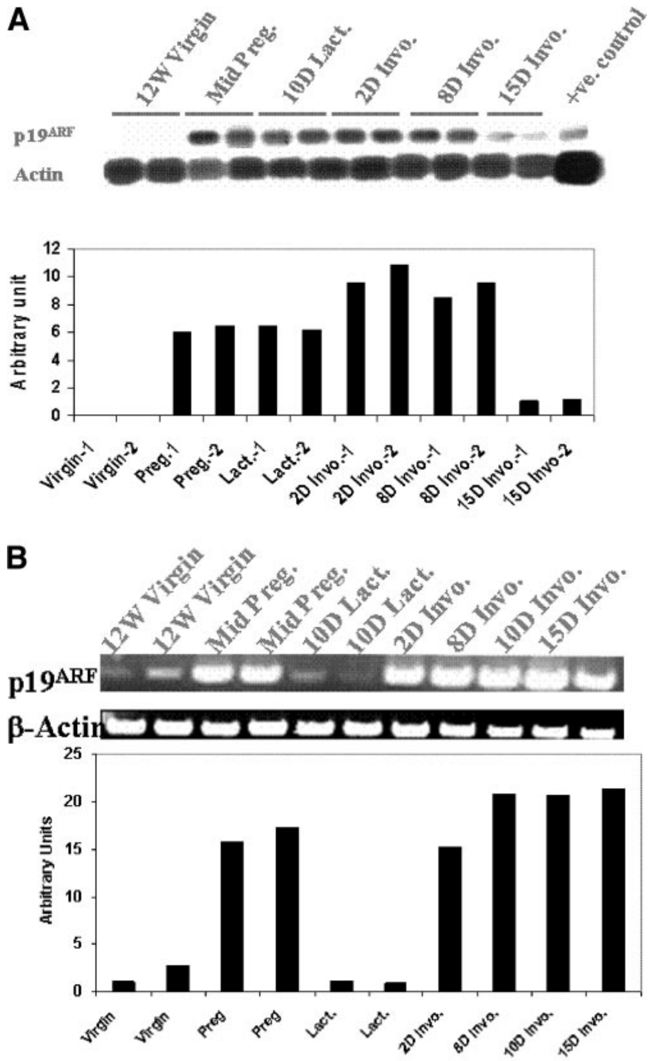


Figure 3. p19^ARF^ expression level during mammary gland development. Mammary glands from WT and p19^ARF^ null mice of 129Svj × C57BL/6 background were analyzed. (A) Protein levels. Equal amounts of protein extracts (500 μg/lane) of WT mammary glands at indicated stage-point during development were analyzed by Western blot for p19^ARF^ and actin levels using rabbit polyclonal antibody specific for mouse p19^ARF^ or actin mAb. Protein bands were detected by ECL detection mixture, scanned, quantified, normalized to the levels of actin, and presented in bar graph. The positive control is BALB/c3T3 10(1) cells. (B) Expression level of p19^ARF^ and β-actin analyzed by RT-PCR on mammary glands of WT and p19^ARF^ null mammary glands at indicated stage-points. Positive control is a PCR product of tail DNA of WT mice.

verse levels of p19^ARF^ message and protein at 10 days' lactation and 15 days of involution may reflect differential p19^ARF^ protein stability at these stages of development.

p19^ARF^ Is Upregulated by Progesterone in Normal Mammary Gland

Because p19^ARF^ was upregulated during pregnancy at both protein and RNA levels, the next question was to identify which hormone or combination of hormones was responsible for the induction of p19^ARF^ expression during pregnancy. Data from a preliminary experiment revealed that 15 days after hormone stimulation with a pituitary isograft is the most appropriate time point to collect mammary glands for estimation of p19^ARF^ protein level (Figure 4A). The pituitary implants constitutively secrets prolactin (Lewis *et al.*, 1986; Singh, 2001). In addition to its direct mammotrophic effects, prolactin exhibits strong luteotrophic effects that induce ovarian corpora lutea to synthesize and secrete progesterone at levels observed in the pregnant mouse (Murr *et al.*, 1974). This mode of hormonal stimulation induces epithelial cell proliferation and alveolar differentiation in the mammary gland similar to that seen during pregnancy (Medina, 1974; Medina and Warner, 1976). p19^ARF^ protein gradually increased at 5 and 11 days and strongly at 15 days after receiving a pituitary isograft (Figure 4A). Results from this preliminary experiment confirmed that p19^ARF^ is upregulated by hormone stimulation. Accordingly, mammary glands were collected 15 days after stimulation with either with E, P, E+P, PRL, or in untreated (control). Data revealed that p19^ARF^ protein level appeared to be unregulated primarily by progesterone alone (Figure 4B). Stimulation by E or PRL alone did not induce p19^ARF^ expression. The p19^ARF^ protein level was undetectable in the control virgin mammary gland (untreated). The increase in p19^ARF^ protein level upon treatment with progesterone alone resulted in an increase in the secondary and tertiary branching, but not alveologenesis (Figure 4B). These data suggest that upregulation of p19^ARF^ protein level by progesterone is not attributed to the increase in number of cells, because mammary glands of both E- or P-treated mice contain adequate number of epithelium and because the actin protein level is similar in all mammary glands exposed to different treatments. Hence, these observations suggest that p19^ARF^ may preferentially be regulated by progesterone in mammary gland during pregnancy to maintain the normal rate of cell proliferation. The twofold increase in the rate of cell proliferation in mammary glands of p19^ARF^ null pregnant mice (Figure 2A) also suggests the p19^ARF^ regulatory involvement in cell proliferation during pregnancy.

To confirm these data and to learn more about p19^ARF^ cellular localization at different hormonal stimulation, double-immunofluorescent staining with p19^ARF^ and K14, a specific protein for myoepithelial cells, revealed that p19^ARF^ is predominantly localized in the luminal epithelial cells in mammary glands that were stimulated with P, and to a lesser extent in those stimulated by E and P (Figure 4C). Results showed no detectable p19^ARF^ was observed in myoepithelial cells of untreated or treated mammary glands by any hormone (Figure 4C). Thus, p19^ARF^ is not normally detectable in neither myoepithelial nor luminal epithelial cells in virgin mammary gland and its expression are detectable in luminal epithelial cells specifically upon P stimulation.

p19^Arf^ Null Mammary Epithelial Cells Are Immortal In Vivo and Have Lost p21^Cip1^ Expression

Compared with wild type, immortalization was demonstrated in mouse embryonic fibroblasts (MEFs) lacking p16^INK4a^, p16^INK4a^/p19^ARF^, or p19^ARF^ alone in vitro (Serrano *et al.*, 1996; Kamijo *et al.*, 1997; Sharpless *et al.*, 2001). Although p16^Ink4a^-null MEFs exhibited an increased rate of immortalization (Sharpless *et al.*, 2001), the rate was less than that for cells null for p16^INK4a^/p19^ARF^, p19^ARF^, or p53 (Serrano *et al.*, 1996; Kamijo *et al.*, 1997). It is not clear whether mammary epithelial cells lacking p19^ARF^ are immortal in vivo. Serial transplantations of p19^ARF^ null mammary epithelial cells demonstrated that the loss of p19^ARF^ is sufficient to immortalize these cells in vivo. Growth potential of WT mammary epithelial cells began to decline in the third trans-

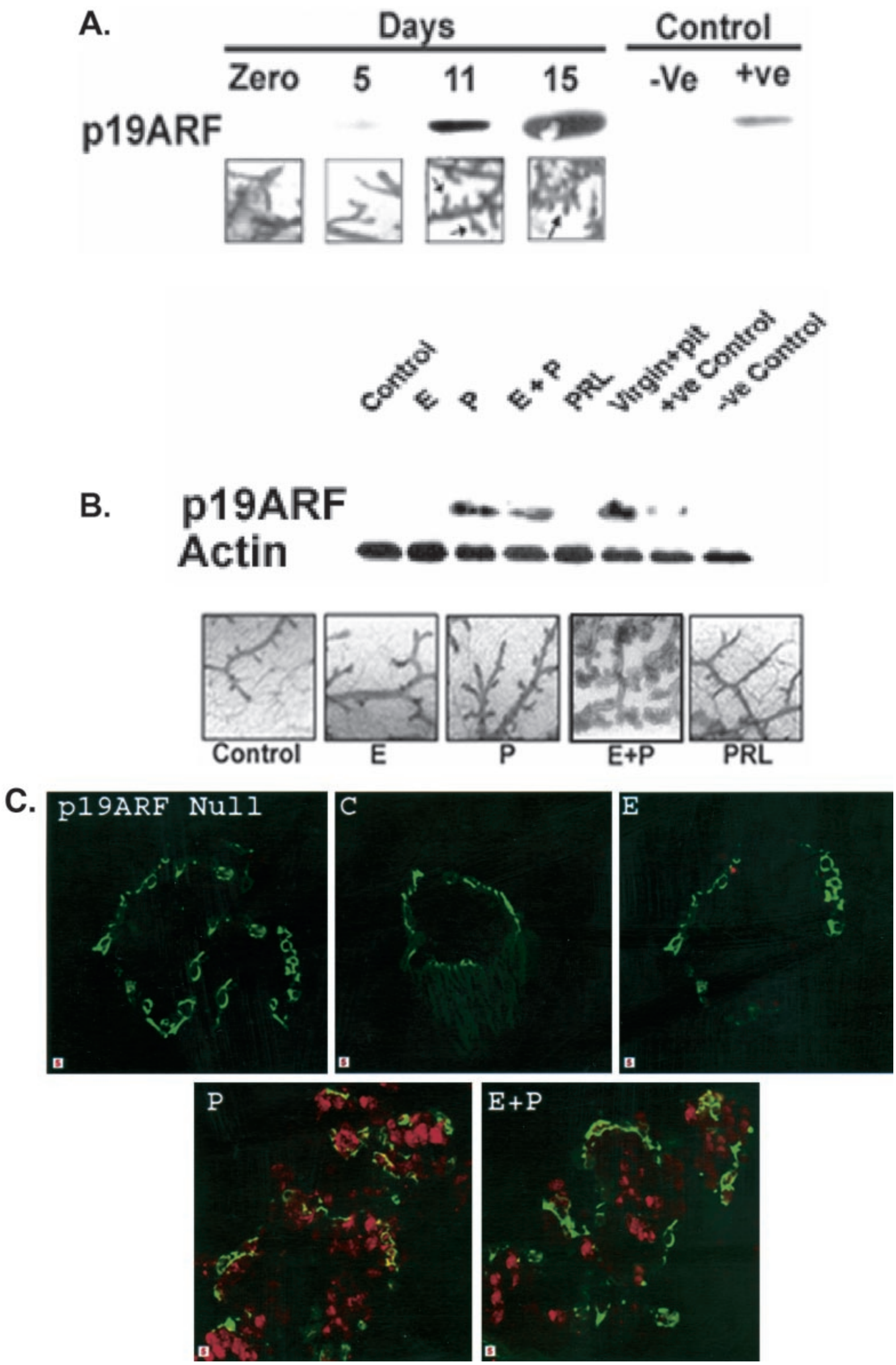
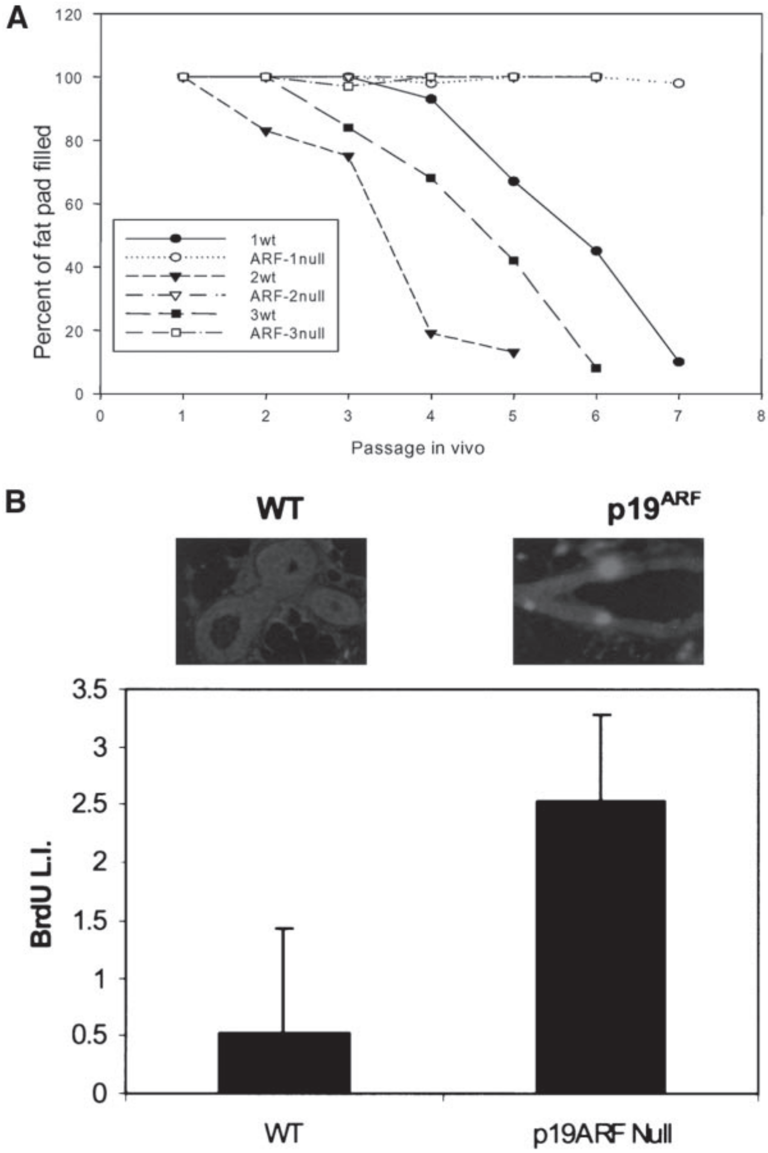


Figure 4. Upregulation of p19^{ARF} and cellular localization in hormonally stimulated normal mammary gland. (A) Western blot analysis of p19^{ARF} protein levels in hormonally stimulated normal mammary glands of virgin WT mice at indicated time points after receiving a pituitary isograft. The positive control and negative controls are protein extract from BALB/c3T3 10(1) (ARF-wild-type, p53-null) cells and 3T3 cells of WT p53, respectively. Below, parts of whole mounts showing changes in morphogenesis at indicated time points. Magnification, ×5. (B) Western blot analysis of p19^{ARF} and actin protein level in mammary glands of mice hormonally stimulated by different hormones as indicated. Also, parts of whole mounts of mammary glands showing change in morphogenesis at individual or combination of hormone stimulation compared with untreated mammary gland (control). (C) Localization of p19^{ARF} in mammary glands at different hormonal conditions. p19^{ARF} stained in red showing overexpression predominantly in luminal epithelial cells in mammary glands stimulated with progesterone, and E+P, whereas weak staining or in PRL or E alone. Two negative controls were included; untreated mammary gland of WT virgin mouse (C) and p19^{ARF} null mammary gland of pregnant mouse. K14, in green, is specifically myoepithelial. Images were taken at (40×) magnification using confocal microscopy.



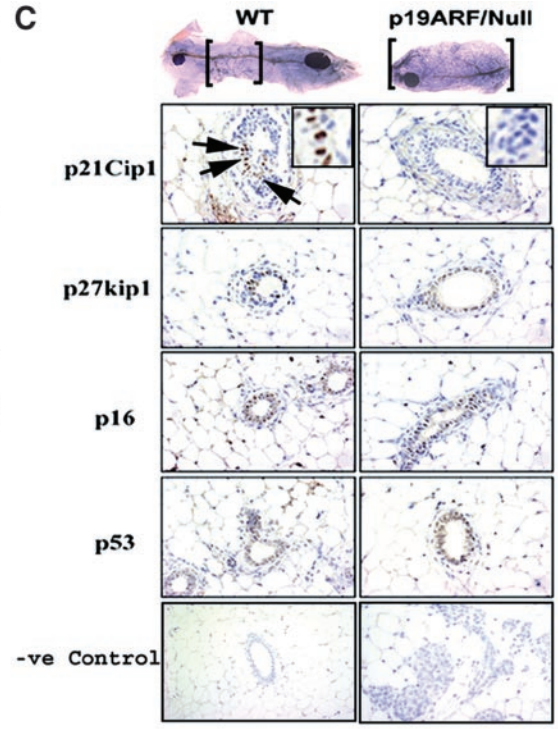


Figure 5. Immortalization of p19^{ARF} null mammary epithelial cells in vivo. (A) A graph presenting percent of area filling the fat pad in 5–6 animals per group for seven transplant generations. The percent of the area filling the fat pad was calculated by measuring the length and width of the ductal area filling the fat pad proportional to the total area of the fat pad using microscope scale. (B) Representative histology sections of WT and p19^{ARF} null mammary outgrowths after six transplants showing BrdU incorporation detected by immunoflorescent staining. A bar graph presenting BrdU labeling index (LI) statistically analyzed from four outgrowths using two-sided Student's *t* test. (C) Whole mounts of the 6th transplant of WT and p19^{ARF} null mammary outgrowths showing the difference in the area filled the fat pad in each cell type. Also, histology sections of the 6th transplant of WT and p19^{ARF} null outgrowths stained for p21^{Cip1}, p27^{Kip1}, p16^{INK4a}, p53, or no primary antibodies (as negative control). Notice the lack of p21^{Cip1}-positive cells in the p19^{ARF} null cells compared with WT in enlarged part of each section.

plant generation, whereas p19^{ARF} null cells continued to totally fill the recipient fat pads beyond the 7th transplant generation (Figure 5A). On the 6th transplant, the difference became greater; only 19% filled the fat pad in the WT compared with the 100% in the p19^{ARF} null outgrowths (Figure 5C), and the BrdU labeling index (LI) was 4.8-fold ($p \leq 0.04$) greater in the p19^{ARF} null cells than in the WT (Figure 5B). These data suggest that mammary epithelial cells lacking p19^{ARF} continue to proliferate indefinitely in vivo in a normal environment, whereas WT cells gradually lose their proliferative capacity and undergo complete replicative growth arrest after six transplants, as expected.

We next asked the question whether the loss of p19^{ARF} has down stream effects on p53, or/and the cdk inhibitors, p16^{INK4a}, p27^{kip1}, and p21^{Cip1}. Immunohistochemical staining of the 6th transplants demonstrated no difference in p53, p16^{INK4a}, and p27^{kip1} protein levels between WT and p19^{ARF} null cells; however, p21^{Cip1} was totally absent in the p19^{ARF} null cells, whereas the WT cells exhibited strong nuclear staining for p21^{Cip1} (Figure 5C). These data clearly suggest that p21^{Cip1} expression is downstream of p19^{ARF} and that lack of p19^{ARF} may play a role in downregulation of p21^{Cip1} expression, possibly through p53 deactivation.

Why the Absence of p19^{ARF} in Mammary Epithelial Cells Delays Involution?

The coordination of signals regulating cell arrest and apoptosis during involution is poorly understood, despite their potential relevance to oncogenesis. The lack of p21^{Cip1} expression in the immortalized p19^{ARF} null cells in vivo (Figure 5C) has encouraged us to investigate whether the delay in mammary involution in the p19^{ARF} null cells is related to downregulation of p21^{Cip1}. Studies demonstrated that p21^{Cip1} expression is induced immediately after weaning, through the p53-dependent pathway leading to acceleration in apoptotic activities (Jerry *et al.*, 1999). Furthermore, overexpression of p19^{ARF} was found to induce p53 activation through acetylation (Korgaonkar *et al.*, 2002), but not through serine 15 phosphorylation (de Stanchina *et al.*, 1998). Thus, it was of interest to examine p21^{Cip1} in p19^{ARF} null

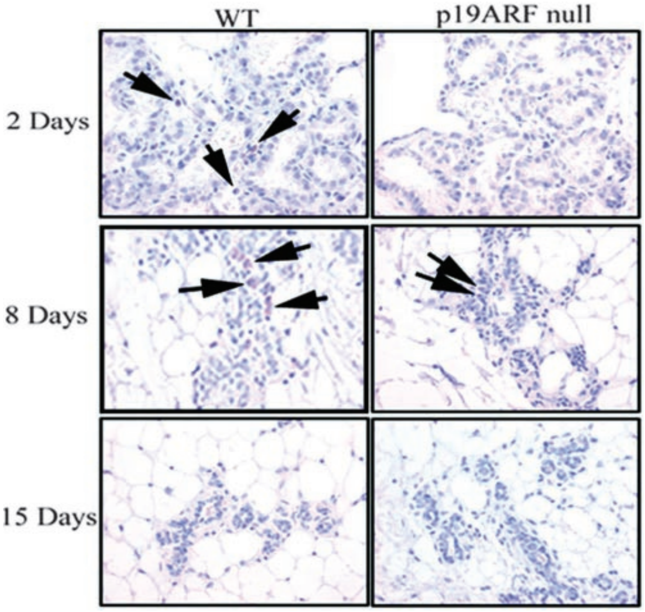


Figure 6. Histology sections of WT and p19^{ARF} null mammary glands during involution stained for p21^{Cip1}. A representative sections of WT and p19^{ARF} null mammary glands at 2, 8, and 15 days of involution stained immunohistochemically for p21^{Cip1} using antibodies specific for mouse p21^{Cip1} (PharMingin Laboratories). Arrows pointed at nuclear and cytoplasmic staining at 2 and 8 days of involution, respectively in the WT, and few nuclear positive cells at 8 days of involution in p19^{ARF} null. Magnification, ×40.

cells during involution. In contrast to the weak to undetectable signal for p21^{Cip1} in the p19^{ARF} null cells at 2 days of involution, strong nuclear positive cells were observed in the WT at same time point (Figure 6). At 8 days of involution, a few moderately positive cells were observed in the WT cells, but were predominantly cytoplasmic, whereas only a few positive nuclear p21^{Cip1} were detected in the p19^{ARF} null cells. p21^{Cip1} protein remained at a low level 15 days after weaning in the p19^{ARF} null cells, but disappeared completely in the WT cells. These data suggest that p19^{ARF} may be an important molecule for p53 activation, which then induces apoptosis through upregulation of p21^{Cip1} expression in early mammary involution. Thus, loss of p19^{ARF} may downregulate p21^{Cip1}, possibly through p53 deactivation that leads to the delay in involution. Further investigation on p53 activity in the absence of p19^{ARF} in early involution is warranted.

DISCUSSION

The mammary gland goes through a rapid ductal proliferation at puberty, followed by the a) proliferative cycle of ductular proliferation and branching with subsequent lobuloalveolar differentiation during pregnancy; b) functional differentiation during lactation with marked decrease in proliferative potential (terminal differentiation); c) a marked increase in cell apoptosis in the initial stage of involution; and d) rapid tissue regression culminating in a return to normal cellularity 7 days after weaning (Fruth, 1999; Jerry *et al.*, 1999).

p19^{ARF} can induce growth arrest through either p53-dependent or -independent pathways. The well studied pathway in p53-positive cells is that ARF sequesters Mdm2 in nucleoli, thereby stabilizing p53 and activating p53-dependent G1 and G2 phase cell cycle arrest (Sherr, 1998; Zhang *et al.*, 1998; Weber *et al.*, 1999). The amino-terminal residues 6–10 and 21–25 of mouse p19^{ARF} were found to be critical functional domains required for p19^{ARF}-induced growth arrest in p53-positive and -negative cells (Korgaonkar *et al.*, 2002).

Our data demonstrated that p19^{ARF} is involved in sustaining the normal rate of cell proliferation during pregnancy and lactation evident by 1) the two- and threefold increases in the rate of cell proliferation, respectively, in the p19^{ARF} null mammary cells compared with WT, 2) the six- and eightfold increases in p19^{ARF} protein and RNA levels, respectively, in mammary glands of pregnant compared with virgin mice, and 3) the upregulation of p19^{ARF} by progesterone. Although, increases in p19^{ARF} at RNA and protein levels during pregnancy confirmed that p19^{ARF} functional role is to control normal rate of cell proliferation, but not differentiation. These data are in agreement with studies demonstrating that p19^{ARF} expression is induced by various mitogenic signals to induce cell growth arrest or apoptosis, depending on the stimulus (Serrano *et al.*, 1997; de Stanchina *et al.*, 1998; Zindy *et al.*, 1998; Cong *et al.*, 1999; Dimri *et al.*, 2000). Therefore, it is conceivable to expect high p19^{ARF} expression level in an extensive proliferative signaling network by the ovarian hormones and growth factors during pregnancy. Surprisingly, one would expect a sharp decline in p19^{ARF} expression and protein levels during lactation as progesterone level drops sharply after perturbation (Shyamala, 1999). Because the decline was only in the p19^{ARF} message level, but not in the protein level during lactation, this data may suggest that progesterone may regulate p19^{ARF} expression level directly or indirectly and that p19^{ARF} protein appears to be stable during lactation. Investigations are ongoing, utilizing PR knockout (PRKO) mice stimulated with progesterone to determine whether p19^{ARF} RNA and protein levels are indeed upregulated by progesterone in vivo. Future experiments will also assess whether progesterone regulates the *ARF* promoter.

The most apparent effect on mammary morphogenesis in the absence of p19^{ARF} was the delay at 2 and 8 days of involution. These data are in contrast to the overexpression of p19^{ARF} at RNA and protein levels at these same time points in the WT mice. These data are in agreement with earlier study on BALB/c mice, where an increase in p19^{ARF} protein at 2 days after weaning was observed, which coincided with the maximal activation of p53-dependent expression of p21^{Cip1}/WAF1 (Jerry *et al.*, 2002). Because these cells are normal, p19^{ARF} is not expected to function through the ARF/MDM2 translocation pathway. In fact, recent studies demonstrated that the growth-suppressive activity of p19^{ARF} does not correlate with the ability to relocalize Mdm2 and that p19^{ARF} can activate p53 through acetylation and inhibit growth without stabilizing p53 (Korgaonkar *et al.*, 2002).

Furthermore, growth-suppressive activity of p19^{ARF} in cells lacking p53 and Mdm2 (Weber *et al.*, 2000b) revealed that other factors besides p53 and Mdm2 can execute p19^{ARF}'s functions. Furthermore, the intensified nuclear p21^{Cip1} protein at 2 days of involution in the WT cells followed by the predominantly cytoplasmic localization at 8 days and complete disappearance at 15 days strongly suggest that the presence of functional p19^{ARF} is necessary to activate p53 to induce p21^{Cip1} expression in the first phase of involution. Data on the p19^{ARF} null cells confirmed the essential functional role of p19^{ARF} in the early days of mammary involution as these cells displayed low p21^{Cip1} expression concomitantly with decrease in apoptotic activity.

These data complement previous studies demonstrated increase in p21^{Cip1} protein level concomitant with increased apoptotic activity during the first 3 days of involution through p53 activation (Jerry *et al.*, 1999).

Furthermore, studies on BALB/c-p53 null showed clear delay in early days of involution that was quantified by the decreases in p21^{Cip1} levels (Jerry *et al.*, 1998). Conversely, C57BL/6-p53 null mice were reported to involute normally (Li *et al.*, 1996); however, the conclusion of the later study was based on one time point at late stages of involution. Furthermore, each study used mice of a different genetic background. It is not clear whether involution is delayed in mammary epithelial cells lacking p21^{Cip1}. It is noteworthy that 8 days of involution appears to be a crucial time point in relation to p19^{ARF} functionality during mammary epithelial cell involution and cellular remodeling. The significant change in apoptosis and cell proliferation at this particular time point during involution can be related to the lack of nuclear p21^{Cip1} in the absence of p19^{ARF}. It is also necessary to detect p19^{ARF} message level at time points beyond 15 days of involution to find out when p19^{ARF} message returns to levels equivalent to that of virgin mammary gland.

Compared with virgin WT mammary gland, the eight- and sevenfold increases in p19^{ARF} message and protein levels, respectively, in involution may suggest another gene that is responsible for the upregulation of p19^{ARF} during involution. One of these genes may be E2F1 (Zhu *et al.*, 1999) or Myc (Zindy *et al.*, 1998; Russell *et al.*, 2002) that has been found to induce apoptosis through upregulation of p19^{ARF} in p53 WT cells. Although controversial data were reported recently on whether E2F1 induces apoptosis through p19^{ARF}-dependent or -independent pathways using different in vitro models (Kowalik *et al.*, 1995), it is necessary to investigate E2F1 expression and activity in p19ARF null cells during involution. In conclusion, p19^{ARF} is preferentially regulated by progesterone during pregnancy to maintain normal cell proliferation rate, but not differentiation. Another regulator, yet to be identified, is responsible for the upregulation of p19^{ARF} during involution to enhance either cell growth arrest or apoptosis during first phase of involution through the p53-dependent pathway.

The correlation between immortalization of p19^{ARF} null cells in vivo and the absence of p21^{Cip1} is novel and clearly suggests that p21^{Cip1} expression is downstream of p19^{ARF} overexpression. This correlation confirmed the critical role of p19^{ARF} in regulation of mammary cell proliferation and apoptosis during development. However, absence of p19^{ARF} does not induce mammary tumors, because one case of mammary adenocarcinoma in p19^{ARF} +/− mice and none in p19^{ARF} null mice were detected (Kamijo *et al.*, 1999). A secondary event is necessary to occur to promote mammary tumorigenesis in p19^{ARF} null mice. An earlier study proposed a possible scenario (Midgley *et al.*, 2000), suggesting that Mdm2 can inhibit p300/CBP-mediated acetylation of p53 (Ito *et al.*, 2001) and that p300 interacts with Mdm2 in a region overlapping the ARF binding site (Grossman *et al.*, 1998; Midgley *et al.*, 2000; Weber *et al.*, 2000a); thus, competitive interaction of ARF to Mdm2 can release p300 to activate p53 through acetylation. Thus, it is essential to examine p53 acetylation sites in p19^{ARF} null mammary gland during involution.

In conclusion, during mammary gland development, the functional role of p19^{ARF} is "partly" responsible to control normal cell growth rate and apoptosis, and the absence of p19^{ARF} leads to immortalization of mammary epithelial cells. The delay in involution in cells lacking p19^{ARF} is partly attributed to the downregulation of p21^{Cip1}, possibly through the p53-dependent pathway.

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